The neutral theory in the genomic era

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A number of tests have been developed to detect positive selection at the molecular level. These tests are based on DNA polymorphism within and divergence between species. Applications of these tests have revealed a large collection of genes that have evolved under positive selection and some general insights into adaptive evolution. Recently, these tests have been applied on a genomic scale and have provided estimates of the frequency of adaptive substitutions and a critical test of the neutral theory.

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Current Opinion in Genetics & Development 2001, **11**:642–646

0959-437X/01/$ – see front matter

Abbreviations

α	proportion of substitutions driven by positive selection
A	amino acid
β	fraction of amino acid polymorphism that is deleterious
ka	rate of amino acid substitution
ks	rate of synonymous substitution
MK	McDonald–Kreitman
N	effective population size
s	selection coefficient
S	synonymous

Introduction

The neutral theory of molecular evolution posits that the majority of DNA variation within and between species is neutral with respect to fitness and can be described by stochastic fluctuations in a finite population [1]. The formulation of the neutral theory provided a number of predictions that could be tested using patterns of DNA polymorphism and divergence [2]. There are two approaches. In the direct approach, amino acid changes are compared to synonymous changes. Because synonymous changes are neutral or nearly neutral with respect to fitness, any difference between patterns of amino acid and synonymous changes within and/or between species can be attributed to selection on amino acid changes. In the indirect approach, selection is detected on the basis of a skew in the frequency distribution of neutral variation linked to a site that has been under selection. The frequency spectrum can also be influenced by a population's demographic history. Positive selection can be distinguished from demographic effects because selection produces a local skew in the frequency spectrum whereas demography produces a genome-wide skew. Although these methods sometimes reveal evidence for positive selection in individual genes, the neutral theory can only be addressed with a genomic estimate of the frequency of positive selection. Recent studies of genomic patterns of amino acid and synonymous polymorphism and divergence have provided insights into the mode and, in some cases, tempo of adaptive evolution.

Divergence

A rate of amino acid substitution (*ka*) greater than that of synonymous substitution (*ks*) is the most robust test for positive selection [3]. A large collection of rapidly evolving genes have been identified by this criteria. The majority are involved in either sexual reproduction or host–pathogen interactions [4•]. This can be explained by the continual improvement in fitness required by sexual selection [5•], and the evolutionary 'arms race' between a host and its pathogen [6•]. The extent to which these genes are reflective of adaptive evolution, however, is not known as even a small amount of constraint on a protein can reduce $ka/ks < 1$.

The power of detecting positive selection is increased through analysis of different codons within a gene [7,8], of different classes of amino acid substitutions [5•,9], and along different phylogenetic lineages [10–12]. By assuming that *ka*/*ks* varies across sites, those codons that are likely to have been under positive selection can be identified [7,13•,14]. Whether approximate or maximum likelihood methods are used, it is clear that mutational and codon biases must be accounted for when estimating *ka*/*ks* [4•,15].

To address the frequency of positive selection in protein evolution, a genomic approach must be taken. For reproductive proteins there is pervasive evidence of positive selection. The rate of divergence of *Drosophila* male and female reproductive proteins is much greater than that of non-reproductive-associated genes [16,17]. Differences in the rate of protein divergence, however, may be caused by differences in selective constraint between the two classes of genes, unless *ka*/*ks* is shown to be >1. This was done in a recent study [18•], which found 11% (19/176) of male reproductive proteins have $ka/ks > 1$ between *D. melanogaster* and *D. simulans*. Previous studies have demonstrated that for some of these genes, the rapid rate of evolution can only be explained by positive selection [19,20]. Male reproductive proteins in primates have also been evolving rapidly; out of 18 male reproductive proteins, positive selection has increased the rate of conservative amino acid substitutions above the synonymous rate in the 11 most rapidly evolving genes [5•].

What fraction of all genes in the genome, not just male reproductive genes, show evidence of positive selection? Using all groups of homologous sequences available in public databases, 0.5% (17/3595) were shown [21] to have $ka > ks$ for more than half of the pairwise comparisons in

the group. More recently, 5.3% (280/5305) of chordate gene families and 3.6% (123/3385) of embryophyta gene families were found to contain at least one branch with a *ka*/*ks* value >1 [22]. More detailed analyses will be needed to place statistical significance on these results and estimate the rate of adaptive substitution.

Polymorphism

Patterns of polymorphism provide another means of detecting positive selection. The spread of an advantageous mutation through a population produces both a reduction in levels of linked neutral variation [23] and a skew in the frequency spectrum [24••]. Positive selection can be detected by a local reduction in levels of variation using the HKA test [25], which compares polymorphism and divergence between two or more regions, or on a genomic level by a positive correlation between levels of variation and rates of recombination. This correlation has been found in a number of species and implies that selection is frequent enough to eliminate most variation from regions of low recombination within the genome (see the review by Aquadro *et al*., this issue [pp 627–634]); however, the elimination of linked neutral variation as a result of background selection against deleterious mutations provides an alternative explanation of the data [26].

The frequency spectrum can be used to distinguish background selection from hitchhiking [27]. Hitchhiking sweeps variation to low and high frequencies [24••], whereas background selection is not expected to produce a skew in the frequency spectrum, at least for small samples sizes [28]. Compared to intermediate frequency variation, both an excess of low frequency variation, as measured by the D statistic, and an excess of high frequency variation, as measured by the H statistic, can be used to detect hitchhiking [24••,29]. Using the H statistic, one out of three regions of low recombination in *D. melanogaster* was found to have a significant excess of high-frequency variants [24••]. Additional evidence for hitchhiking comes from the correlation found between rates of recombination and the D statistic [30•], as is expected under a model of recurrent hitchhiking [31]. Because the combined action of both positive and negative selection cannot easily be disentangled [32•], it would be difficult to obtain an estimate of the frequency of adaptive substitutions on the basis of a reduction in levels of variation [33].

With large-scale surveys of polymorphism, positive selection can be detected by scanning regions of high recombination for a local reduction in levels of neutral variation accompanied by a skew in the frequency spectrum. This method has been used in the MHC region of humans [34] and in the *D. melanogaster* genome using microsatellite variation [35,36]. Similarly, a local reduction in levels of variation surrounding the newly evolved *Sdic* gene in *D. melanogaster* was used as evidence for positive selection at this locus [37]. Linkage disequilibrium mapping uses levels of phenotypic and genotypic associations to simultaneously detect selection and map the mutation responsible for the phenotype. This has been used to map the warfarin resistance gene in rats [38••], and the desaturase gene responsible for a cuticular hydrocarbon pheromone polymorphism in *D. melanogaster* [39•]. Linkage disequilibrium has been examined on a genomic scale in humans to identify regions that are likely to have been influenced by selection [40].

Table 1

Estimates of the fraction of low frequency amino acid polymorphism that is deleterious (β) and the fraction of amino acid substitutions that were driven by positive selection (α).

Data	A/S			β	α	Refs.
	Rare	Common	Fixed			
H. sapiens	1.11	0.57	0.88	0.48	0.35	[42•]
D. melanogaster	0.63	0.29	0.63	0.54	0.54	(a)
D. simulans		0.06	0.20		0.70	[57]
D. simulans complex		0.07	0.21		0.68	[58]

(a) JC Fay *et al*., unpublished data.

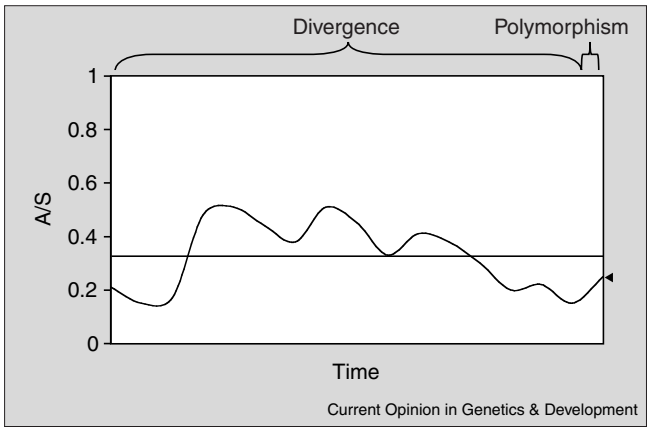
Positive selection can also be detected by a skew in the frequency spectrum of amino acid compared to synonymous variation. The largest polymorphism survey with outgroup sequence [41], which is necessary to distinguish low and high frequency mutations, shows a larger A/S ratio at high (7/7) compared to intermediate frequencies (28/38), as expected if some amino acid mutations were advantageous [42•]. With more data, the strength and frequency of adaptive mutations could be estimated.

Polymorphism and divergence

The McDonald–Kreitman (MK) test has the advantage of being able to detect positive selection in the presence of a strong selective constraint [43]. The test compares the ratio of the number of amino acid (A) to synonymous (S) polymorphic sites to the A/S ratio of fixed differences between species. If a large fraction of amino acid substitutions are driven by positive selection, the A/S ratio of divergence should be inflated above that of polymorphism. Although the power of the MK test is clearly much greater than *ka*/*ks* [44], it is based on several assumptions that are often not justified. By comparing polymorphism and divergence from a large number of genes these assumptions can be either removed or tested, as discussed below.

The proportion of amino acid substitutions driven by positive selection can be estimated from the difference between the A/S ratio of common polymorphism and divergence (the use of common polymorphism is discussed below). For all species with data from a large number of genes, the estimate is surprisingly high (Table 1); however, these estimates assume synonymous mutations are neutral and the constraint on a gene remains constant over time. Although there is evidence for selection on synonymous

Figure 1



Hypothetical ratio of neutral amino acid (A) to synonymous (S) mutations that become fixed along a lineage. The A/S ratio of divergence is a measure of the average amount of constraint on a gene and is the cumulative number of amino acids divided by synonymous substitutions over time, as shown by the horizontal line. The A/S ratio of polymorphism is a measure of the selective constraint on a gene in the recent past, as shown by the arrowhead.

sites in *Drosophila* [45], codon bias is not correlated with the rate of synonymous substitution [46••,47]. This indicates that a change in the strength of selection on synonymous sites would be too weak to produce a change in the A/S ratio. A change in selective constraint on the protein sequence is a more serious consideration.

The assumption that the selective constraint on a gene is constant, as measured by A/S, is rarely justified. The constraint on a gene is determined by the fraction of amino acid altering mutations for which $2Ns < -1$, where N is the effective population size and s is the selection coefficient [3]. Fluctuations in either population size or the strength of selection are expected to produce fluctuations in the A/S ratio of mutations that become fixed along a lineage (Figure 1) [48,49]. By chance, the A/S ratio of extant polymorphism may be lower than that of divergence, which is a measure of the average A/S ratio between two species. Although fluctuations in the intensity of selection may either increase or decrease the A/S ratio of a single gene over time, only a change in effective population size is expected to produce a genome-wide effect on the A/S ratio. Regardless of the cause, the magnitude of the fluctuation in the A/S ratio depends on the distribution of Ns values associated with amino acid mutations. If a large proportion of mutations have Ns values that lie close to –1, then the A/S ratio is expected to be quite sensitive to even slight changes in N or s.

The nearly neutral theory of molecular evolution places an emphasis on the role of slightly deleterious amino acid mutations in explaining patterns of DNA variation [50]. The original support for the theory came from the large excess of allozyme variation found at low frequencies within human and *Drosophila* populations [51]. Demographic explanations have now been ruled out by showing there is an excess of low frequency amino acid compared to synonymous polymorphism ([42•]; JC Fay *et al*., unpublished data). The difference between the A/S ratio of rare compared to common polymorphism was used to obtain estimates in both *Homo sapiens* (181 genes) and *D. melanogaster* (31 genes) of the fraction of amino acid polymorphism which is slightly deleterious (Table 1). Slightly deleterious amino acid mutations may lie approximately between $-100 < 2Ns < -1$, but are simply defined as those mutations with significant effects on levels of polymorphism (>1%) and rates of divergence. By fitting the data to a population genetic model, it was estimated that 20–40% of amino acid mutations are slightly deleterious in humans and the average individual is expected to carry over 500 of these mutations in their genome [42•].

The large fraction of amino acid mutations that have been estimated to be slightly deleterious have two important implications for applications of the MK test. The first is that the A/S ratio of polymorphism is inflated by slightly deleterious amino acid mutations segregating at low frequencies in a population. This effect can be removed, at least in a crude manner, by comparison of the A/S ratio of common polymorphism with divergence [42•,45,52]. The second implication is that the A/S ratio may be particularly susceptible to changes in effective population size. Given a reduction in effective population size, the A/S ratio of subsequent substitutions may approach that of low-frequency variation, which is quite high (Table 1). Therefore, a high A/S ratio of divergence may be accounted for by a period of reduced effective population size.

A change in population size can be distinguished from positive selection by comparison of the A/S ratio of rare and common polymorphism to that of fixed differences (JC Fay *et al*., unpublished data). A change in effective population size should affect all genes, and the degree to which each gene is affected should depend on the fraction of amino acid mutations in that gene which are slightly deleterious. Thus, a change in population size predicts a correlation between the A/S ratio of divergence and that of low-frequency polymorphism. On the other hand, positive selection should not affect all genes to the same extent. This approach was taken in the analysis of polymorphism in *D. melanogaster* and divergence to *D. simulans* (JC Fay *et al*., unpublished data). The difference between 11 rapidly evolving genes, which were found to account for the entire difference between the A/S ratio of polymorphism and divergence, and 34 slowly evolving genes was found to be compatible with positive selection but not a change in effective population size. The slowly evolving genes were found to have an excess of low frequency amino acid variation but only a slight excess of amino acid divergence, whereas the rapidly evolving genes were found to have a large excess of both low-frequency polymorphism and divergence compared to common polymorphism (JC Fay *et al*., unpublished data).

Additional theoretical and empirical research will be needed to refine estimates of the fraction of amino acid mutations that are both deleterious, neutral and adaptive. The human polymorphism and divergence data contain different genes and the *Drosophila* data are limited by inconsistent sampling. An unbiased sample of 50 or more genes from a species whose history is unrelated to human demography is needed. The comparison of the A/S ratio of low and common polymorphism to that of fixed differences allows for only a rough estimate of the impact of slightly deleterious and adaptive mutations on protein polymorphism and divergence. Optimally, a maximum likelihood method, which incorporates mutational and codon usage biases, should be used in the analysis of polymorphism and divergence data [53]. Although some theoretical work has been done [48,49,54,55•,56], empirically the effect of a change in population size on the rate of molecular evolution is not well understood but is most pertinent to our understanding of molecular evolution.

Conclusions

The analysis of DNA polymorphism and divergence on a genomic scale will provide critical tests of the neutral theory of molecular evolution. Application of the MK and other A/S ratio based tests have provided substantial evidence that positive selection has inflated the rate of protein divergence above that expected on the basis of polymorphism. Future studies will need to estimate the relative contributions of changes in population size and positive selection to this pattern. The role of positive selection in the divergence of duplicate genes and in the evolution of gene regulatory elements has yet to be addressed.

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